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THE EFFECTS OF OVERMATURITY ON EGGS OF
RANA TEMPORARIA

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THE EFFECTS OF OVERMATURITY ON EGGS OF RANA TEMPORARIA

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ABSTRACT: Discusses the effect of overmaturity of eggs on the sex ratio of resulting *Rana temporaria*, and shows that the cytoplasm is the only part affected.

Richard Hertwig's investigations on sex determination in frogs have shown that maturity of eggs promotes the formation of males. The sex ratio was shifted in favor of the male with increasing period of time between the first, normal fertilization and the following (artificial) fertilization. Kuschakewitsch finally succeeded in increasing the percentage of males from 53 to 100% by inducing hypermaturity of eggs of 89 hours. The objection previously raised repeatedly viz. that the different results obtained in fertilizations carried out at different moments were due to the greater mortality of the females has been rendered invalid by the fact that mortality in his cultures did not surpass 4 to 6%. His results, thus, demonstrate that a purely male progeny will result once overmaturity of eggs has reached a certain degree. Hertwig furthermore has demonstrated that employment of aged, overmature sperm does not influence the sex ratio. Overmaturity, thus, affects the eggs only, and these must therefore undergo certain changes conferring on them the tendency for forming males. The question then arises whether these changes chiefly affect the cytoplasm or the nuclear material of the egg. Hertwig initially assumed the latter possibility to be true believing that the formation of the polar body was affected by overmaturity of the egg. That such an influence may, in fact, be exerted is demonstrated by the observation that even in highly overmature eggs the polocyte of the second division is aborted only after fertilization, i. e., after evacuation into the water. For the elucidation of the question in which manner polar body formation proceeds under the influence of overmaturity, one would have to know whether the male or the female represents the heterogametic sex in the frog. Unfortunately we are as yet not fully informed about the chromosomal make-up of this species. For that reason, Hertwig has offered explanations covering both cases, and I wish to repeat them briefly.

The action mechanism of overmaturity could best be explained by the assumption of the existence of heterogamy in the female. In that case, the numbers of eggs containing an "X" chromosome and those not containing this chromosome would be equal under normal circumstances. The spermatocytes, however, would all exhibit the same constitution and contain the "X" chromosome. On fertilization the eggs containing the "X" chromosome would form 50% of

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*Numbers in the margin indicate pagination in the foreign text.

homogametic males, and the eggs not containing the "X" chromosome would form 50% of heterogametic females. These latter ones would thus be missing in the cultures of overmature eggs. If we assume that the course of meiosis is modified by overmaturity of the eggs in this manner, i.e., that the "X"-free chromosomal set enters the polar body destined for elimination, then there would remain only eggs containing "X" chromosomes, which, in turn, would lead to the formation of a purely male, homogametic progeny.

It has, however, been demonstrated that in all vertebrates, whose spermiogenesis has been investigated more closely, the female animal is the homogametic one and the male the heterogametic one, and there exist no reasons to suppose the opposite to be true in frogs. By representing all autosomes by a dash, the chromosomal formula of the male will be $-x-o$, and for the female of the species $-x-x$. This means that all eggs would contain the "X" element. Half of the spermatocytes would, however, contain the "X" chromosome, while the other 50% would be characterized by its absence. Normal fertilization would, thus, lead to the formation of the former complement number viz. 50% homogametic females and 50% heterogametic males. If overmaturity, however, would influence meiosis in a manner leading to the elimination of the two "X" chromosomal elements with the polocytes, then eggs exhibiting the formula $-o$ would be formed only. Such eggs would on fertilization form 50% of individuals exhibiting the chromosomal formula $-x-o$ and 50% of individuals exhibiting the formula $-o-o$. The former would be normal, heterogametic males, and the latter would be individuals—probably males—characterized by a total absence of the "X" element. The question, however, is whether such individuals would, in fact, be viable. /99

In the case that one of these two hypotheses would prove to be correct, we would not be dealing with true sex transformation. This change would rather represent a shift of the sex ratio due to the fact that only one gamete type is formed, while the other one (in our case, the polocyte) perishes, as in the case (in a similar manner) in male aphides. This means that only males should develop in cultures of overmature eggs, and that no indifferent intersex forms should develop which is in contrast to the normal process in frogs. Should we, however, succeed in demonstrating the presence of indifferent forms in such cultures, which forms would change into males in the subsequent course of development, then we would indeed have evidence indicating the occurrence of a sex transformation due to overmaturity of the eggs produced by a specific influence exerted on the cytoplasm. Gehalt Rat von Hertwig has personally informed me that he has changed his previously held views because of results obtained by him in more recent experiments. He has kindly made his unpublished manuscripts available to me, and he has summarized the results of his recent investigations in the following sentences:

"1. Overmaturity of the eggs accelerates differentiation of the testes. While it is not possible to differentiate with a certain degree of certainty between males and females in normal cultures even after metamorphosis, characteristic testicular tissue can be demonstrated at an early stage in at least half of the culture material obtained from overmature eggs. This finding corresponds to one repeatedly obtained in normally grown frog material viz. that one half of the individuals exhibits testicular tissue, while the other half—apparently the half destined to become females—exhibits gonadal tissue showing no differentiation.

2. The results obtained in the cultures mentioned disprove the interpretation of overmaturity effects previously given by me viz. that overmaturity causes changes in the course of egg development; that the female of this species represents the heterogametic sex; that the sex chromosome enters the polar body in the course of egg development by which mechanism male-producing eggs only would be formed."

Since Hertwig's experiments were carried out only with *Rana esculenta*, he asked me last spring to investigate the situation existing in *Rana temporaria*.

Before reporting on the results of my experiments, I should like to expand /100 on a description of the methods employed because *Rana temporaria* proved in many respects to be poorly suited for experiments concerning the investigation of overmaturity of eggs. This fact was probably due not only to the object itself but also to the poor animal material made available to me as well as to the unfavorable weather conditions prevailing this year during spawning-time. The greater part of the frogs employed in my experiments came from the region around R hrmoos, a small village near Dachau north of Munich. They were captured in copula and transported separately. Upon arrival at the institute, the frogs were put into large containers where they soon copulated again, whereupon they were immediately isolated. I made a great mistake by obtaining my experimental material from a frog catcher, who probably kept the animals separated, and a part of them stored for a certain period of time before delivering them to me. Due to this handling, the possibility of overmaturity of the eggs was given a priori since one cannot depend on assurance given to the contrary.

One third of my material consisted of animals, which had passed the winter at the institute. Since *Rana temporaria* commences spawning after a period of fasting, these frogs are better suited for experiments on the effects of overmaturity than those captured in the field, because the time of copulation can be determined exactly in these animals.

The first copulating frogs were captured on March 8th. Spawning reached a peak between March 13th and 18th and it ceased toward the end of the month. The partners were isolated and put into rectangular glass containers filled one hand high with water. In order to give these animals the opportunity to reach a dry surface, the containers were either tilted or a few bricks were put into them. The water was replaced every few days by fresh water adapted to room temperature. The rooms containing the aquariums were as far as possible protected from disturbances, and in some instances the containers were covered with cloth. Since *Rana temporaria*, in contrast to *Rana esculenta*, spawns within a very short time (after copulation), close surveillance of the animals was required. Despite all efforts, I did not always manage to catch the moment of deposition of eggs in order to separate the animals at that moment. A further difficulty presented itself by the fact that most frogs spawned at night. Of the 42 frog couples employed in my experiments, only 13 spawned during the day, 26 during the night, and three copulating partners separated without deposition of eggs taking place. It thus became necessary to expand surveillance to include the nights. The chief difficulty, however, arose only after I had succeeded in separating the mating partners at the correct moment viz. immediately after the deposition of a small number of eggs, since the female even after interrupted copulation continued in almost all instances to deposit the eggs still remaining in the uterus. In order /101

to prevent such a deposition, I later on put the females into dry containers and set these immediately into running water of 10°C. By this procedure of inducing cold and providing a dry environment, I hoped to prevent a further deposition of eggs. But even in this attempt I did not always succeed, and when I finally sacrificed the animals in order to obtain eggs for the purpose of artificial fertilization, I was once again disappointed when I opened the abdomen in the hope of finding a portion of eggs remaining in the uterus. In addition, I never did succeed in inducing partners once separated to engage in a second or, indeed, a third act of copulation and to obtain in this way overmature eggs for culturing following natural fertilization, as was achieved repeatedly with success by Hertwig in experiments with *Rana esculenta*. My cultures of overmature eggs were, thus, obtained by artificial fertilization. How this is done has already been described in some detail by Richard Hertwig. For the purpose of identifying the various cultures, I employed colored glass beads, a method introduced by von Hertwig, which has proved to be an extraordinarily practical one. Breeding of the larvae was carried out according to the old approved method described by me in a previous paper. The animals were preserved in sublimate-glacial acetic acid and stored in 70% alcohol. This method of fixation also proved to be very suitable for the microscopic examination of gonadal tissues.

The duration of copulation varied to a relatively great extent. This, however, is said to have no effect on the sex ratio. Copulation usually lasted for four to six days. One set of mating partners separated after five days without the female having spawned. Upon opening of this female, I found that the eggs were still in the ovary. Another set of partners separated after 7 days of copulation, and the female spawned alone during the next night. These eggs, of course, died since they were not fertilized. Examination of the female revealed that she had deposited all her eggs, and no eggs at all were found in either the uterus or the ovary. This observation demonstrates that in *Rana temporaria* eggs once descended into the uterus usually are deposited without the cooperation of the male, a fact which impedes the induction of overmaturation to an extraordinary degree as mentioned above.

Deposition of the eggs, as mentioned above, takes place within a relatively short period of time. How long this process, in fact, lasts was not ascertained by me since I was solely interested in separating the partners once deposition of eggs had commenced. Eggs are not deposited in a number of small portions, as is the case in *Rana esculenta*, but in one single, large and shapeless ball. This ball first sinks to the bottom (of the tank) and later, once the gelatine covering has swelled, the ball rises to the surface of the water. However, it frequently occurs that the gelatine surrounding the eggs situated in the middle of this rather compact ball does not swell or at least does not swell to the same extent as does the gelatine closer to the surface of the ball, in which way a solid nucleus of closely packed eggs remains in existence in the middle of the ball. Development of the larvae as a rule proceeds at a much slower rate in the interior of the ball than in the surface layers, which no doubt is due to the decreased supply of oxygen in the interior. /102

In one culture, all eggs deposited on March 13th were well orientated indicating proper fertilization. By March 16th, tripartite embryos had developed in the surface eggs, while eggs in the interior exhibited an early blastula stage only. On the next day, the former had hatched while the latter had only reached

the late blastula stage. Eggs in the intermediate regions exhibited various stages of embryonal development depending on the proximity to the surface. Finally a part of the eggs situated in the most unfavorable region died and this frequently after having reached a rather advanced stage of development. In studies in the field, I have observed that this sequence appears to be the rule under natural conditions. Balls of eggs collected in the field developed in the laboratory in the manner just described. It is, however, possible to induce uniform development by cutting the ball of eggs into smaller pieces using a scissor for this purpose.

Despite the large material on hand at the outset of my investigation, I was able to obtain a total of only two cultures of overmature eggs. The first one, which I designed culture No. 1, was derived from a couple of mating frogs captured in Røhrmoos. They were isolated on March 13th. On March 17th, at 4 p. m., the animals were separated following the deposition of a small ball of eggs. Approximately 40% of the eggs derived from this first, normal fertilization eventually hatched and the animals thus obtained were used for breeding purposes in the laboratory.

The female was immediately exposed to cold (10°C) in a dry tank. Despite these precautionary measures, she deposited a number of additional eggs by herself. When I sacrificed this animal on March 20th at 11 a. m., it was found to contain a number of eggs in the uterus, which number was large enough for artificial fertilization. Approximately 20% of these eggs began development and were used for culturing. Overmaturity amounted in this case to 67 hours.

Culture No. 2 was the product of two laboratory animals, which had copulated on March 21st. They were separated on March 24th once deposition of eggs was noted. Approximately 30% of the eggs deposited at that time developed. In this instance, too, the female deposited a great number of additional eggs despite the taking of the precautionary measures mentioned. When I sacrificed this animal on March 29th, exactly 5 days (120 hours) after separation, only a relatively small number of eggs remained in the uterus for the purpose of artificial fertilization. Sixty-two tadpoles developed from the 173 eggs obtained, representing a total of 26%; only 14 of these tadpoles reached the stage of metamorphosis. Mortality was, thus, very great in culture No. 2, and the capacity for development was considerably reduced by 120 hours of overmaturity.

In addition to the two cultures just described, I breed six cultures obtained following normal fertilization in order to investigate the sex ratio in general and the occurrence of undifferentiated intersex form in particular. /103

The proper determination of the sex in frogs is an extraordinarily difficult undertaking. The external shape of the gonads varies greatly due to the occurrence of numerous transitional forms, and one frequently is not certain whether the gonad under examination is one as yet undifferentiated or a female or male one, respectively. Even microscopic examination of the tissue does not always aid in obtaining reliable results. Only long experience will eventually permit correct diagnosis. In this connection, I may mention an observation made by Witschi, which I can confirm and which in certain doubtful cases may be of decisive importance: "The testes of *Rana temporaria* exhibit a certain characteristic viz. the peritoneum covering the testes may contain pigmented cells.

Unfortunately these pigment cells are not found in all instances; otherwise determination of the sex would be an easy matter. These pigmented cells, which can be seen through the peritoneum are never found in ovaries."

The following points may be made about the gonads: Animals exhibiting long-stretched, lath-shaped gonads of minimal thickness have been found to be indifferent types. These gonads never exhibit either the moniliform shape (which has been described as being characteristic for the indifferent form of *Rana esculenta*) or central cavities causing cystoid protrusions of the germinal epithelium. Microscopic investigations have revealed that these cavities are small and cleft-like, and they do not affect the appearance of the externally smooth and even surface of the gonads (Figure 1). The tendency for the formation of a male was indicated by a concentration of the gonadal mass at the cranial pole and by a tendency for the formation of the characteristic spindle- and club-shaped testes, which, as mentioned above, may be pigmented, in which case they are easily recognized (Figure 2). The ovaries were either thick and cylindrical with a smooth surface (Figure 3) or lobulated and folded exhibiting a number of contractions (Figure 4). The first mentioned form of ovary represents a particular type, about which more is said further below. I should furthermore like to mention that the right gonad usually develops at a faster rate than the left one, a fact also observed in *Rana esculenta*. This difference in developmental rate can be seen particularly well in larvae just undergoing metamorphosis. In instances like these, it was found that the right gonad had developed into a characteristic testis, while the left one still exhibited an indifferent character.

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The table (Table 1 of this translation) shown gives a summary of the sex ratios found in the two cultures of overmature eggs.

TABLE 1

	1.	2.
I.	18 ♀ 2 J 23 ♂	2 ♀ 11 J 26 ♂
	67	
II.	2 ♀ 9 J 2 ♂	0 ♀ 12 J 2 ♂
	120	

The Roman numbers I and II denote the two cultures; the Arabic numbers denote the two instances of fertilization between which the extent of overmaturity is given in hours. The sex ratio exhibited by the animals developed after the first fertilization of culture No. 1 eggs is a largely normal one, and the number of males is only slightly larger than that of females. As expected the number of males surpassed that of females to a considerable extent following the second fertilization; however the indifferent intersex forms also significantly increased in numbers. Their number had increased from 5% to 39% of the total number of animals. This

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Figure 1.
Indifferent
Gonad.

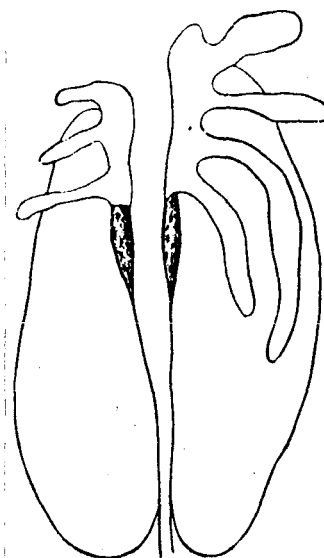


Figure 2. Character-
istic Testis Showing
Pigmentation

fact may perhaps be explained by a process in which the males of cultures of overmature eggs develop out of indifferent individuals through transformation.

We would then in this instance be dealing with such forms undergoing transformation, which accordingly would have to be more numerous in this than in normal cultures. The fact that an extensive transformation into males was actually taking place is indicated by the frequent appearance of animals exhibiting a right gonadal gland which had already developed into a testis, while the left one showed an indifferent character. This view is supported in particular by a comparison of the results obtained following sacrifice of the culture material at certain intervals; the results are shown in the next table (Table 2 of this translation).

These results show clearly that the number of indifferent forms decreased with the age of the culture in favor of the males. There can be no doubt that a transformation of forms originally having a female direction into males is, indeed, taking place so that the entire culture material in the end is male. The impetus for this metagenetic transformation apparently can be found in the overmaturity of the eggs. I should like to mention that the two females of the first sacrifice exhibited gonads which I, on macroscopic inspection, held to be ovaries. A microscopic study, however, revealed that they were, in fact, undergoing transformation into testicular tissue, so that I could have listed these two animals in the column showing the number of indifferent forms.

Culture No. 2 exhibited a markedly indifferent character right from the start, and the great number of indifferent forms developing following the first fertilization is evidence for this particular character. The numbers obtained, however, are so small that it is not admissible to draw conclusions from them. These numbers, however, do not contradict my views elaborated above, since the two males developed after the second fertilization belonged to the group sacrificed last. The finding of such a high number of indifferent intersex forms may

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perhaps be explained by the fact that the first group—which consisted of intersex forms only—was sacrificed earlier than was the case in culture No. 1. The transformation, then, was not yet advanced as far as that observed in culture No. 1.

The demonstration of indifferent forms in my cultures of overmature eggs and their gradual disappearance shows that sex is not immutably predetermined

TABLE 2

Fixed on	♀	J	♂
May 16	2	4	4
May 26-28	0	4	12
May 30-31	0	3	10

by the formation of only one gamete type. We are dealing here rather with a metagenic alteration of the sex, a process previously observed in other animal species. I am thinking in this connection of the situation in *Bonellia viridis*. This species also exhibits indifferent forms which may then develop into either males or females. Depending on whether the larva attaches itself to the proboscis of the mother or not a male or a female animal will develop. In this instance, the originally female character of the larva is metagenetically changed by the adoption of a parasitic mode of life. In frogs the development direction toward the fe-

male is changed in a similar sense by overmaturity. Overmaturity directly affects the gonad products only; in fact, as previously demonstrated by the results obtained by Hertwig, it affects the eggs only. We are now in a position to add a further qualification viz. that only the cytoplasm of the egg is affected by overmaturity of the egg. The fact that aged sperm does not affect the sex ratio is in good agreement with this view. Spermatozoa contain practically no cytoplasm at all, and they can therefore not be influenced by factors affecting cytoplasm, and this in particular since the small amount of cytoplasm present in spermatozoa plays no role at all in development. If we now make the assumption that in frogs—like in other amphibians and all vertebrates until now investigated in this regard—the male sex is the heterogametic one, i. e., that the male contains only one "X" chromosome, then the second heterochromosome must perish in the metagenetic transformation of originally female oriented animals into male ones. The chromosomal make-up is apparently corrected in this sense by a secondary process occurring in the cytoplasm affected by overmaturity.

Hertwig, whose investigations in *Rana esculenta* have led to similar results, wrote in the (unpublished) manuscript already mentioned:

"This situation (i. e., the alteration of the sex in frogs) reminds one of processes known to occur in certain hermaphroditic species, in which initially a development of homogametic females takes place, which in the course of further development acquire the capacity for forming testicular tissue by a process during which a part of the gonadal cells lose one of the two "X" chromosomes present. I would interpret the situation observed to have developed in the same way. I assume that the male of the species frog is heterogametic, as is asserted by others for the frog—on the basis of not entirely reliable observations—as well as for other amphibians and vertebrates. Consequently two types of eggs must arise following fertilization: so-called "male eggs" containing one "X" chromosome, and "female eggs" containing two "X" chromosomes. The latter undergo an alteration in the course of development in a way during which the second "X"

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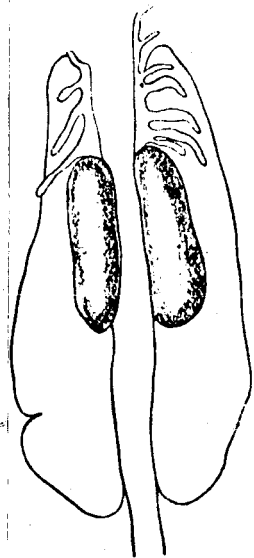


Figure 3. Cylindrical Ovary
(Cultures Nos. VI-VIII).

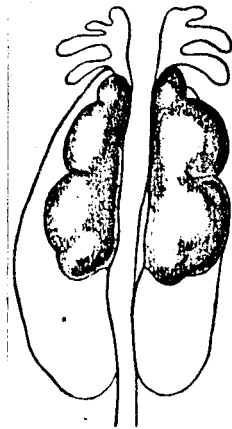


Figure 4. Characteristically Lobulated
Ovary (Magnified 15 Times. Drawn with
the Aid of Abb's Drawing Apparatus).

chromosome either regresses completely or is weakened functionally. I think it probable that the influences causing the alteration of the chromosomal make-up are exerted by the cytoplasm, as is the case in hermaphroditic forms, and not only in these but also in animals like daphnids and aphids, in which a transition from the female to the male sex also takes place in the course of the generation sequence."

I am not able to state by which mode of action overmaturity may affect the cytoplasm of the egg; investigations on the mode of action are presently under way at this institute, and these may help to elucidate the problem.

Before closing, I should like to discuss the matter of sex ratios in other, that is normal frog egg cultures since they permit the drawing of certain conclusions. In the table shown above (Table 3 of this translation), I have summarized some numerical data pertaining to this matter. These data were obtained in freshly metamorphosed froglets.

Culture No. 3 exhibited a very normal character, and the sex ratio obtained in this culture corresponded largely to that found following the first fertilization of culture No. 1 eggs. Only three indifferent forms were found, while all other animals could be easily differentiated, and sex determination presented no difficulties. This culture differs from the other cultures in that all the females obtained exhibited the characteristic lobulated ovary type, one of which is shown in Fig. 4. Cultures Nos. 4 and 5 were characterized by a large excess of males.

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Apparently we were in these cases from the start dealing with overmaturity for the reasons outlined above. Culture No. 5 exhibited almost indifferent forms,

while the remaining cultures exhibited very remarkable sex ratios.

These ratios were characterized by a marked excess of females. Culture No. 7 contained females only.

The ovaries of all these animals exhibited the peculiar type shown in Fig. 3. They were cylindrical with a smooth surface. Hertwig has described the same kind of ovaries in cultures of *Rana esculenta* exhibiting females only. He was able to demonstrate that such cultures develop when the sex products undergoing fertilization had the tendency to develop into indifferent forms. This observation permits the conclusion that in this instance the parents produced eggs and spermatozoa, respectively, both exhibiting an indifferent tendency. It has turned out that indifference is not caused by external factors coming into effect during development but that there exist local strains, the progeny of which exhibits an indifferent character, and other strains, the progeny of which is characterized by an early sex differentiation. The frogs taken in Röhrmoos thus represent an "indifferent" strain, and in this respect they were suitable for my experiments.

TABLE 3

Cult. No.	Sex ratio			Origin
III.	37 ♀	3 J	42 ♂	Röhrmoos
IV.	9 ♀	8 J	26 ♂	Röhrmoos
V.	1 ♀	30 J	8 ♂	Röhrmoos
VI.	32 ♀	16 J	4 ♂	Institute
VII.	16 ♀	—	—	Röhrmoos
VIII.	30 ♀	1 J	1 ♂	Röhrmoos

spectively, both exhibiting an indifferent tendency. It has turned out that indifference is not caused by external factors coming into effect during development but that there exist local strains, the progeny of which exhibits an indifferent character, and other strains, the progeny of which is characterized by an early sex differentiation. The frogs taken in Röhrmoos thus represent an "indifferent" strain, and in this respect they were suitable for my experiments.

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